THE SOIL AS A SOURCE OF MICROORGANISMS ANTAGONISTIC TO DISEASE-PRODUCING BACTERIA*,[1]

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Bacteria pathogenic for man and animals find their way to the soil, either in the excreta of the hosts or in their remains. If one considers the period for which animals and plants have existed on this planet and the great numbers of disease-producing microbes that must have thus gained entrance into the soil, one can only wonder that the soil harbors so few bacteria capable of causing infectious diseases in man and in animals. One hardly thinks of the soil as a source of epidemics. What has become of all the bacteria causing typhoid, dysentery, cholera, diphtheria, pneumonia, bubonic plague, tuberculosis, leprosy, and numerous others? This question was first raised by medical bacteriologists in the eighties of the last century. The soil was searched for bacterial agents of infectious diseases, until the conclusion was reached that these do not survive long in the soil. It was suggested that the cause of the disappearance of these disease-producing organisms in the soil is to be looked for among the soil-inhabiting microbes, antagonistic to the pathogens and bringing about their rapid destruction in the soil.

Pasteur (Pasteur and Joubert, 1877) deserves the credit for having first shown, in 1877, that the production of anthrax in susceptible animals can be repressed by the presence of other microorganisms ("... on peut introduire à profusion dans un animal la bactéridie charbonneuse sans que celui-ci contracte le

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charbon: il suffit qu'au liquide qui tient en suspension la bactéridie on ait associé en même temps des bactéries communes"). It was soon established that when anthrax, typhoid, staphylococcus, diphtheria, and other bacteria are added to the soil they are rapidly antagonized by the soil microbes. Such common saprophytes as *Bacterium fluorescens* and *Bacterium pyocyaneum* were found (Garré, 1887; Bouchard, 1889; Freudenreich, 1888) to produce substances, antagonistic to the colon-typhoid group of bacteria as well as to many others. The degree of inhibition varied with the organisms antagonized. Frost (1904), who was among the first to make a detailed study of the rôle of soil organisms in repressing the development of pathogens, found that the saprophytes produce substances which not only inhibit the growth of the pathogens, but bring about their destruction. The nature and activity of these thermostable substances were found to depend not only upon the nature of the antagonist, but also upon the specific strain, the composition of the medium and the conditions of growth, especially aeration.

An extensive literature has accumulated on this subject. The numerous antagonistic organisms so far described can be divided into four distinct groups:

The first group comprises bacteria belonging to *Pseudomonas aeruginosa* (*Bacterium pyocyaneum*), *Pseudomonas fluorescens*, and related forms. Marked differences in ability to antagonize bacteria appear to exist among the various strains. The specific nature of the active substance has received a great deal of consideration, various claims having been made that it was an enzyme, a pigment, or a lipoid. Hettche (1934) has shown that it passes through collodion membranes, as well as through Seitz and other filters, and is of a lipoid type.

The second group of antagonists comprises various spore-forming bacteria, belonging to the *Bacillus mycoides* and *Bacillus mesentericus* groups. These organisms were found (Pringsheim, 1920; Much and Sartorius, 1924) to bring about lysis of a number of pathogenic bacteria, including diphtheria, typhoid and cholera. In most cases, the antagonistic organisms seemed to be highly specific, acting only upon certain bacteria and not upon others. Recently, Dubos (1939) isolated from the soil an organism belong-

ing to the spore-forming bacteria, which produces a substance capable of destroying gram-positive bacteria; the active agent was crystallized and shown to be a protein derivative. Hettche and Weber (1939), on the other hand, reported the isolation of an active lipoid from spore-forming bacteria. This substance appears to be far more specific than that obtained by Dubos.

A third group of antagonists comprises certain actinomycetes. Some of these organisms inhibit the multiplication of bacteria, whereas others are capable of lysing dead and living bacteria (Lieske, 1921; Gratia and Dath, 1924–1926; Welsch, 1937–1939; Borudulina, 1935). An active substance, which seemed to be largely antagonistic against gram-positive bacteria, was produced (Gratia and Dath, 1924–1926; Nakhimovskaia, 1937). Although this substance was designated as "actinomycetin" (Welsch, 1937–1939), it is fairly certain that different species produce substances which vary in their action.

A fourth group of microorganisms, the fungi, also comprises a number of forms which produce substances active against bacteria, such as "penicillin" obtained by Fleming (1929), and against fungi, as in the case of *Trichoderma* species (Weindling, 1932–1936).

This brief survey is sufficient to emphasize the fact that various microorganisms are capable of forming substances which either inhibit bacterial growth or are bactericidal, or both. These substances vary greatly in composition, in the nature of the organisms which they antagonize, and in the mechanism of the process of inhibition or bactericidal action. Most of the antagonistic organisms appear to act upon gram-positive bacteria, although many also antagonize gram-negative forms. We report certain preliminary results of a study of the nature and abundance of some soil microorganisms, with special reference to their action against various bacteria, comprising both gram-positive and gram-negative forms.

METHOD OF DEMONSTRATING ANTAGONISTIC MICROORGANISMS IN SOIL

The following method has been developed for determining the presence in the soil of microorganisms capable of antagonizing

various specific bacteria. Agar is washed in distilled water and dissolved so as to give 1.5 per cent concentration. Two grams of K_2HPO_4 are added per liter. Ten-milliliter portions of this agar are distributed in test tubes and sterilized. A washed suspension of the specific bacteria, obtained by cultivation on solid or in liquid nutrient media is prepared and added to the washed agar, which has previously been melted and placed in a water bath at 42°C. One-milliliter portions of the still viable bacterial suspension are added to the agar tubes. The bacteria are thoroughly mixed with the agar.

The soil to be tested for the presence and abundance of the antagonists is suspended in sterile tap water, using a series of dilutions, from 1:10 to 1:10,000. One-milliliter portions of these dilutions are placed in sterile Petri dishes and the bacterial agar prepared by the above procedure is added. The plates are well shaken, to distribute the soil suspension thoroughly, and incubated at 28° or 37°C. The presence of antagonists can be demonstrated by the formation of clear zones surrounding the colonies of the latter, after 1–10 days' incubation of the plates. These colonies can now be transferred to fresh bacterial agar plates and later isolated in pure culture by the use of convenient media (figs. 1 and 2).

In the case of certain soils or of specific bacteria, antagonists may not be present at all or they may be there only in limited numbers, not sufficient to give distinct colonies on the plate. Recourse may then be had to the soil enrichment method. Washed bacterial suspensions, for which antagonists are to be obtained, are added, at frequent intervals, to a fresh garden or field soil, kept in beakers or pots under optimum conditions of moisture (60 to 65 per cent of the water-holding capacity), aeration and temperature (28° or 37°C.). The soil is tested from time to time until antagonists can be demonstrated by the plating method.

Attention may be directed here to the fact that the measurement of the concentration of bacteriophage in bacterial cultures is based upon the use of suspensions of live bacteria in agar. Lieske (1921) and Gratia and Dath (1924–1926) suspended

bacteria in agar in order to obtain antagonistic microorganisms, especially actinomycetes; however, they first killed the bacteria

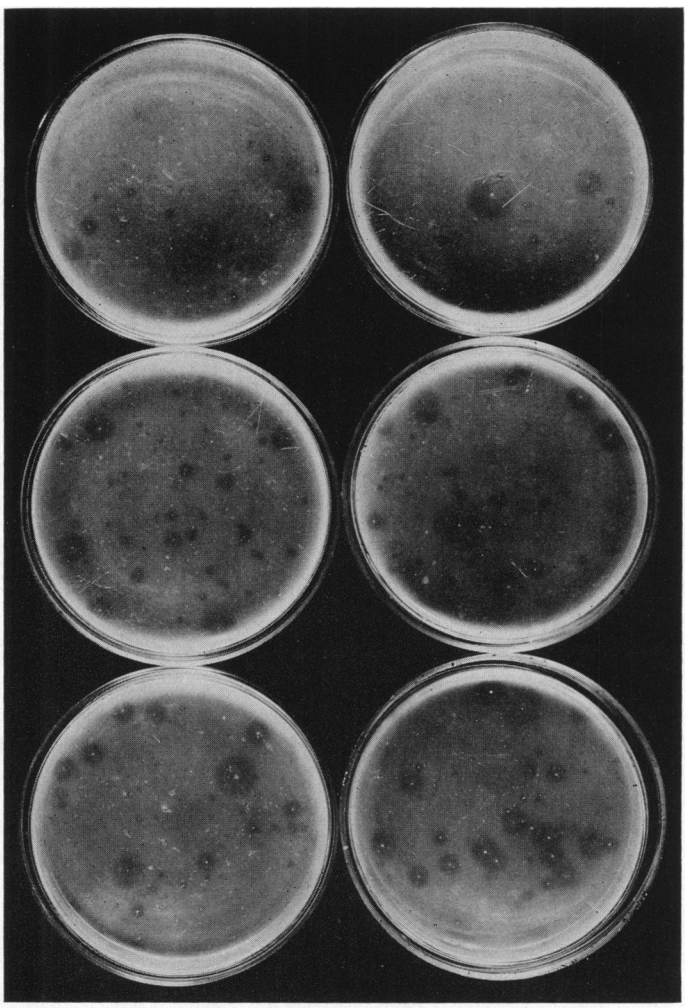

FIG. 1. METHOD OF ISOLATION OF ANTAGONISTIC MICROORGANISMS FROM SOIL
E. coli used as the enriching agent. Plates one day old

by the use of heat or chemicals, and exposed the plates to the air in order to catch chance antagonists in the dust. Schiller (1924–

1925) stimulated various organisms to develop antagonistic properties by using specific bacteria as the sole source of nitrogen.

By means of the method outlined here, it was possible to demonstrate that ordinary soils contain a number of microorganisms which are antagonistic against various bacteria, including both gram-negative and gram-positive forms. The number of antag-

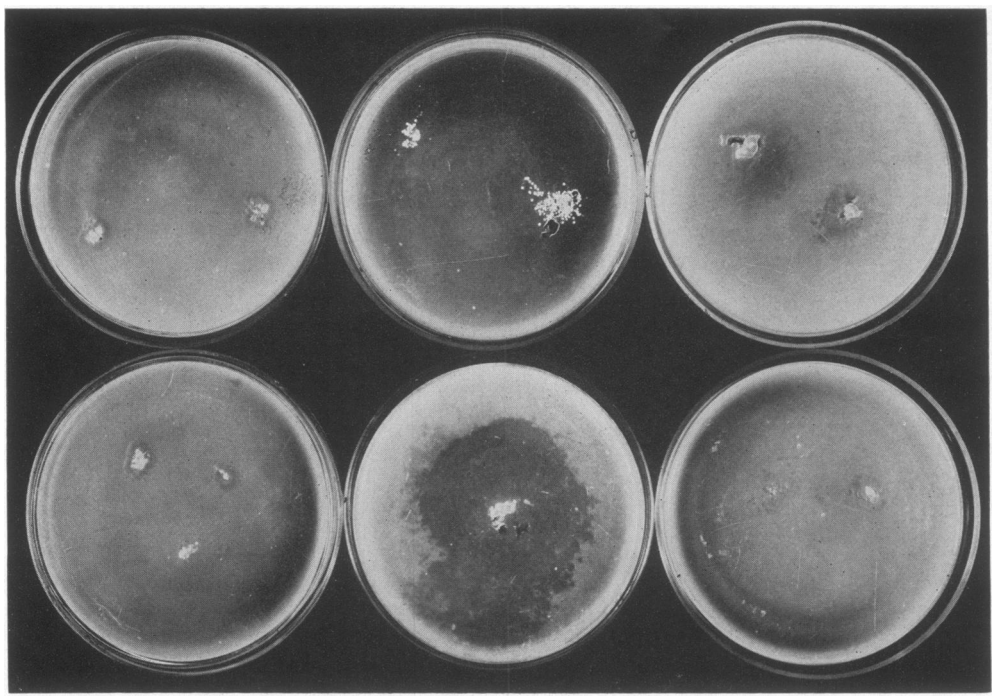

FIG. 2. ISOLATION OF ANTAGONISTS FROM PLATES
Left two plates—first isolation. Middle two plates—nearly complete clarification. Two plates at right—reisolation of cultures.

onists was greatly increased in the soil by gradual enrichment with live cells of the specific bacteria which were to be antagonized. A number of antagonistic organisms were isolated.

This report will be limited, however, to a discussion of only two antagonists: (1). a gram-negative organism representing a strain of *Pseudomonas aeruginosa;* (2). a species of *Actinomyces* be-

longing to the chromogenous type, producing black pigments on protein media.

ISOLATION OF ANTAGONISTIC MICROORGANISMS FROM SOIL

In order to demonstrate the effect of enrichment of soil with specific bacteria upon their disappearance and upon the accumulation of organisms antagonistic to them, the results of one experiment are reported (tables 1 and 2). *Escherichia coli* was used for enrichment purposes. The physical and chemical soil conditions had no injurious effect upon this organism, since it

TABLE 1

Survival of Escherichia coli in sterile and in non-sterile soil

Thousands per gram of soil

STERILE SOIL			FRESH SOIL		
Incubation (28°C.)	*E. coli*	Incubation (28°C.)	No lime	$CaCO_3$	No lime
days		*days*	*28°C.*	*28°C.*	*37°C.*
0*	2.6	0	Few§	Few	Few
10	149,000	5†	6,800	3,500	4,700
26	138,000	33†	130	140	10
		127‡	0	1.1	0

* 2,600 cells of *E. coli* added per 1 gram of sterile soil.

† One enrichment before second count; five enrichments before third count.

‡ Eleven enrichments before fourth count, ten days elapsing between last enrichment and plating.

§ Very few colon-like cells in fresh soil (less than 10,000 per gram, practically all of the *Aerobacter aerogenes* type).

not only survived in sterile soil but actually multiplied there at a very rapid rate. The addition of several hundred living cells of *Escherichia coli* to 1 gram of sterile soil (260,000 per 100 grams of soil) resulted in an increase, in 10 days at 28°C., to 149 millions per gram of soil. This tends to prove that the soil, freed from other organisms, is a rather favorable medium for the multiplication of *Escherichia coli*, and that some of the soil organic matter is available to this organism as a source of energy, or may have been made available by the sterilization of the soil. However, the enrichment of fresh soil with large numbers of living *Esch-*

erichia coli cells led to their rapid disappearance; the rate of their destruction increased with every subsequent addition of fresh bacterial cells. The destruction of the *Escherichia coli* was brought about by the development of certain antagonistic microbes which began to multiply rapidly in the enriched soil (table 2). The high number of antagonists even in the control soil may have been partly due to contamination of some of this soil kept in the laboratory, with *Escherichia coli* cells, which resulted in an enrichment of antagonists. When fresh field soils were tested, a much smaller number of antagonists was found in one and only a

TABLE 2

Influence of enrichment of soil with Escherichia coli upon the abundance of antagonists

INCUBATION	CONTROL SOIL	ENRICHED SOIL	ENRICHED SOIL + $CaCO_3$
	Numbers of antagonists* per 1 gram of soil		
days			
65	500,000	4,000,000	6,000,000
117	1,150,000	5,700,000	4,700,000†
Total numbers of microorganisms capable of developing on agar plate			
117	9,100,000	40,000,000	36,300,000

* An antagonistic colony is one surrounded by a halo on the *E. coli* plate.

† This container received fewer enrichments with *E. coli* than the one without $CaCO_3$.

few in another. The total number of bacteria in the enriched soil, as determined by the plate method, increased very greatly, because the cells of *Escherichia coli* served as good nutrients for many of the soil microorganisms, especially the antagonists.

Several of the organisms isolated were active against the coliform group of bacteria and *Brucella abortus*, in addition to various gram-positive bacteria. Particular emphasis was laid upon a member of the *Pseudomonas aeruginosa* group and a species of *Actinomyces*. These two organisms were grown on a peptone solution (1 per cent Bacto tryptone + 0.5 per cent NaCl), in shallow layers, at 37°C. and 28°C. respectively, for 5 to 10 days.

In the case of the *Actinomyces*, it was sufficient to filter the culture through paper; in the case of the bacterium, a Seitz filter was used to obtain bacteria-free preparations. These were added, in varying concentrations, to sterile nutrient agar media and the inhibiting effect upon various bacteria was tested.

Table 3 shows the inhibiting and bactericidal effect of *Pseudomonas aeruginosa* upon the growth of two test bacteria. *Escherichia coli* was killed rapidly, as demonstrated by streaking the culture on an Endo plate, even when inoculated 1–3 days before

TABLE 3

Bactericidal effect of Pseudomonas aeruginosa upon Escherichia coli and Aerobacter aerogenes

CULTURE	PRELIMINARY INCUBATION*	P. AERUGINOSA†	SURVIVAL OF		
			E. coli	*A. aerogenes*	*P. aeruginosa*
	days				
E. coli	0		+		
+	0	+	0		+
+	1	+	0		+
+	2	+	Trace		+
+	3	+	0		+
A. aerogenes	0			+	
+	0	+		Trace	+
+	1	+		Trace	+
+	2	+		Trace	+
+	3	+		Trace	+

* Incubation of cultures, at 37°C., before inoculation with antagonist.
† Total incubation, at 37°C., 6 days; 0 = no growth; + = positive growth.

the antagonist was added to the culture. The destructive effect upon *Aerobacter aerogenes* was not as marked. When the Seitz filtrate of *Pseudomonas aeruginosa* was added to sterile nutrient agar (table 4), it completely inhibited the growth of *Sarcina lutea* in concentration of 1:20 and reduced it in 1:100 concentration; it inhibited the growth of *Bacillus mycoides* and of *Brucella abortus* in 1:10 concentration. However, the filtrate did not inhibit the growth of *Escherichia coli*, even in 1:5 dilution, but merely reduced it somewhat. Repeated experiments demonstrated definitely that, although the presence of living cells of

Pseudomonas aeruginosa was sufficient to inhibit the growth and even kill *Escherichia coli*, the bacteria-free filtrate had no effect upon this organism, although it had a decided effect upon the other three bacteria tested.

An attempt was now made to obtain the active substance in concentrated form. Various reagents were used for extraction

TABLE 4

*Inhibiting effect of Seitz filtrate of Pseudomonas aeruginosa upon the growth of four test bacteria**

AMOUNT OF FILTRATE ADDED TO 10 ML. AGAR	E. COLI	B. MYCOIDES	S. LUTEA	B. ABORTUS
cc.				
0	3	3	3	3
0.1	3	3	1	3
0.5	3	3	0	3
1.0	3	0	0	0
2.0	2	0	0	

* 0 = no growth, 1 = trace, 2 = fair, 3 = good growth.

TABLE 5

Inhibiting effect of the ether-soluble fraction of Pseudomonas aeruginosa upon the growth of three test bacteria

AMOUNT ADDED TO 10 ML. NUTRIENT AGAR	E. COLI	B. MYCOIDES	S. LUTEA
*cc.**			
0	3†	3	3
0.05	3	3	3
0.10	3	3	0
0.50	1	0	0
2.00	0	0	0

* 1 cc. contained 0.4 mgm. of active fraction.

† 0 = no growth, 1 = trace of growth, 2 = fair growth, 3 = good growth.

and concentration, until it was demonstrated that ether extracted most of the active substance. This fraction was found to be highly effective in inhibiting the growth of *Sarcina lutea*, *Bacillus mycoides* and *Escherichia coli*, in increasing concentrations (table 5). It inhibited the growth of *Sarcina lutea* in 1:100 dilution (0.04 mgm. of crude active substance per 10 ml. agar), of *Bacillus*

mycoides in 1:20, and of *Escherichia coli* in 1:5 dilution (0.8 mgm. of substance per 10 ml. agar); it reduced considerably the growth of *Escherichia coli* in a 1:20 dilution (figs. 3 and 4).

The results of a study of the effect of the composition of the medium upon the production of the active substance are given in table 6. The reaction of the medium, as a result of the growth of

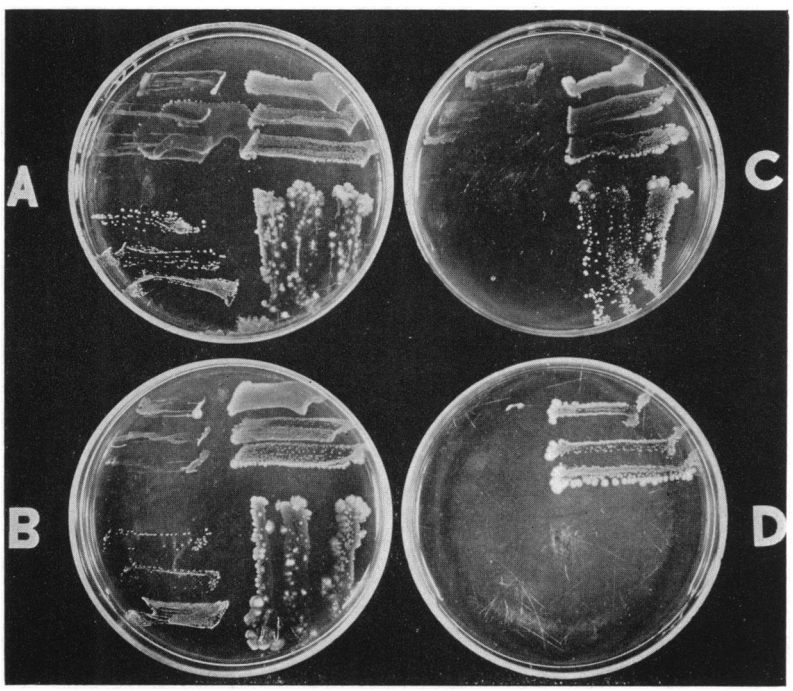

FIG. 3. INFLUENCE OF ETHER-SOLUBLE FRACTION OF P. AERUGINOSA UPON THE GROWTH OF FOUR BACTERIA

A—Control. B, C, D—0.05, 0.2 and 1.0 cc. per plate, respectively. Upper right—*E. coli;* upper left—*B. abortus;* lower right—*B. mycoides;* lower left—*S. lutea.*

Pseudomonas aeruginosa, changed from neutral to pH 9.0; the presence of glucose increased the growth of the organism but did not affect the final pH value. The addition of glucose to the culture reduced its effectiveness; however, it increased the activity of the ether-soluble substance. This is due to the fact that the whole culture, including the thick, slimy growth mass, was ex-

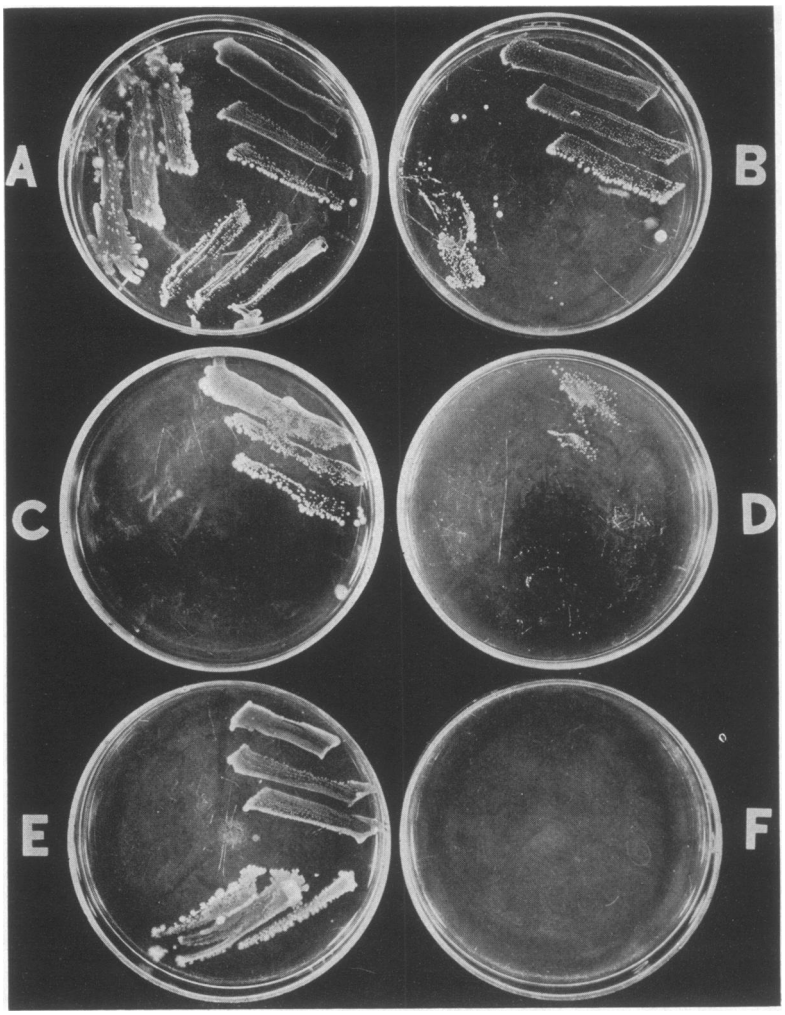

FIG. 4. COURSE OF FORMATION OF ACTIVE ETHER-SOLUBLE SUBSTANCE BY PS. AERUGINOSA

A—Control; B—1 day incubation, 2 cc.; C, D—2 days incubation, 0.5 and 2.0 cc.; E, F—7 days incubation, 0.1 and 2.0 cc. All except E: upper right—*E. coli;* upper left—*B. mycoides;* lower—*S. lutea.* E: upper right—*E. coli;* upper left—*S. lutea;* lower—*B. mycoides.*

tracted with ether; a good deal of the active substance was retained by the cells, growth being heavier on the glucose-containing media. In the presence of *Escherichia coli*, which made an

effective growth for 24 hours, *Pseudomonas aeruginosa* did not develop so well, especially in the presence of glucose; this is due to the fact that *Escherichia coli* used up a large part of the nutrients and changed the reaction of the medium to acid, in the presence of glucose. As a result of this, in spite of the fact that the *Escherichia coli* was killed, the antagonist produced only a

TABLE 6

*Influence of composition of medium upon the formation of the inhibitory substance by Pseudomonas aeruginosa**

Tryptone broth as basis

ORGANISM	GLUCOSE ADDED	FINAL pH	AMOUNT OF FILTRATE USED	ACTIVITY‡ OF FILTRATE				ACTIVITY‡ OF ETHER SOLUBLE FRACTION		
				S. lutea	*B. mycoides*	*E. coli*	*B. abortus*	*S. lutea*	*B. mycoides*	*E. coli*
			cc.							
P. aeruginosa	0	8.8	0.5	1	3	3	3	0	0	3
P. aeruginosa	0	8.8	1.0	0	0	3	0	0	0	2
P. aeruginosa	+	8.5	0.5	2	3	3	2	0	0	3
P. aeruginosa	+	8.5	1.0	0	1	3	2	0	0	0
E. coli† + *P. aeruginosa*	0	8.9	0.5	3	3	3	3	0	2	3
E. coli + *P. aeruginosa*	0	8.9	1.0	3	3	3	3	0	0	3
E. coli + *P. aeruginosa*	+	4.5	0.5	3	3	3	3	0	2	3
E. coli + *P. aeruginosa*	+	4.5	1.0	3	3	3	3	0	1	3

* Active substance 20 times more concentrated in ether-soluble fraction than in original filtrate.

† Inoculated with *E. coli* 24 hours before inoculation with *P. aeruginosa*, incubated at 37°C.; total incubation period 7 days.

‡ 0 = no growth, 1 = trace of growth, 2 = fair growth, 3 = good growth.

limited amount of active substance; the latter could be demonstrated only in the ether-soluble fraction.

The inhibiting action of the *Actinomyces* sp. upon the growth of the three test bacteria was similar in nature but greater in effect (table 7). The paper filtrate of the culture inhibited the growth of *Sarcina lutea* in a dilution of 1:200, of *Bacillus mycoides* in 1:100, but did not inhibit the growth of *Escherichia coli*. Heating the filtrate for 30 minutes at 100°C. did not reduce its

activity. The ether-soluble fraction, in very great dilution, completely inhibited the growth of all the test organisms. This active fraction may be designated as *actinomycin*, in contradistinction to the water-soluble actinomycetin of Gratia (1924–1926) and Welsch (1937–1939).

Further studies brought out the fact that many of the actinomycetes of the soil are antagonistic to different bacteria. Some were found to be more effective against the colon group of bacteria, while others were more effective against the *Brucella* group. It was also demonstrated that the coliform group could

TABLE 7

Inhibiting effect of Actinomyces sp. upon the growth of three test bacteria

AMOUNT ADDED TO 10 ML. NUTRIENT AGAR	PAPER FILTRATE OF ACTINOMYCES SP. CULTURE			HEATED FILTRATE OF* ACTINOMYCES SP. CULTURE			ETHER-SOLUBLE FRACTION† OF ACTINOMYCES SP. CULTURE		
	E. coli	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*
cc.									
0	3‡	3	3	3	3	3	3	3	3
0.01	3	3	3						
0.05	3	3	0						
0.10	3	0	0						
0.50	3	0	0	3	0	0	0	0	0
2.00	3	0	0	3	0	0	0	0	0

* Heated for 30 minutes at 100°C.

† 1 cc. = 0.4 mgm. of active substance.

‡ 0 = no growth, 3 = good growth.

be differentiated on the basis of the action of the antagonist: *Escherichia coli* was most readily acted upon, *Aerobacter aerogenes* least readily and the intermediate strains came between, as shown by the following summary[2]:

ACTINOMYCIN ADDED TO 10 ML. NUTRIENT AGAR	GROWTH OF		
	E. coli	Intermediate	*A. aerogenes*
mgm.			
0.4	0	0	2
0.8	0	0	0

[2] The authors are sincerely indebted to Griffin and Stuart (1940) for supplying these cultures.

The specific action of the antagonists against these organisms may offer an explanation for the common observation that *Escherichia coli* dies out rapidly when added to the soil, whereas *Aerobacter aerogenes* becomes established in the soil.

The inhibiting action of the active ether-soluble substance isolated from the antagonistic Actinomyces grown on agar media was found to be, in the case of the three test organisms, as follows: A dilution of 1:2,500,000 inhibited the growth of *Sarcina lutea* and of *Bacillus mycoides*, and 1:25,000 inhibited the growth of *Escherichia coli* (fig. 5).[3]

Bactericidal action of active substances. Attention has already been called to the fact that the two antagonists studied here have a marked bactericidal effect upon various gram-positive and gram-negative bacteria. This action is not limited to the presence of living and multiplying cells of the antagonists, but is also brought about by the formation of certain bodies which can be isolated from solid and liquid cultures of the antagonists. These active bodies are adsorbed by charcoal and are dissolved by ether. By the use of the latter, followed by treatment with alcohol, then with water, an aqueous solution of the active substances is obtained. This solution had a marked bactericidal effect upon *Escherichia coli* and *Brucella abortus*, as brought out in table 8. The tests were made by adding various dilutions of the active substance to 10 ml. of sterile water containing a suspension of living *Escherichia coli* cells grown on agar media, removed, centrifuged, washed with water and again centrifuged. The apparent increase in the number of cells as a result of addition of very dilute solutions of the active substance may be due to the separation of the flocculated material which otherwise tends to settle to the bottom.

Similar tests were made with *Brucella abortus*. A suspension containing 34,000,000 viable cells was treated with varying

[3] Further studies brought out the fact that the inhibition of the growth of *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus hemolyticus* and the pneumococcus was of the same range as of *Sarcina lutea*; the inhibition of species of *Neisseria* and of *Mycobacterium tuberculosis* was of the range of *Brucella*, whereas that of the typhoid-paratyphoid group was of the range of *Escherichia coli*.

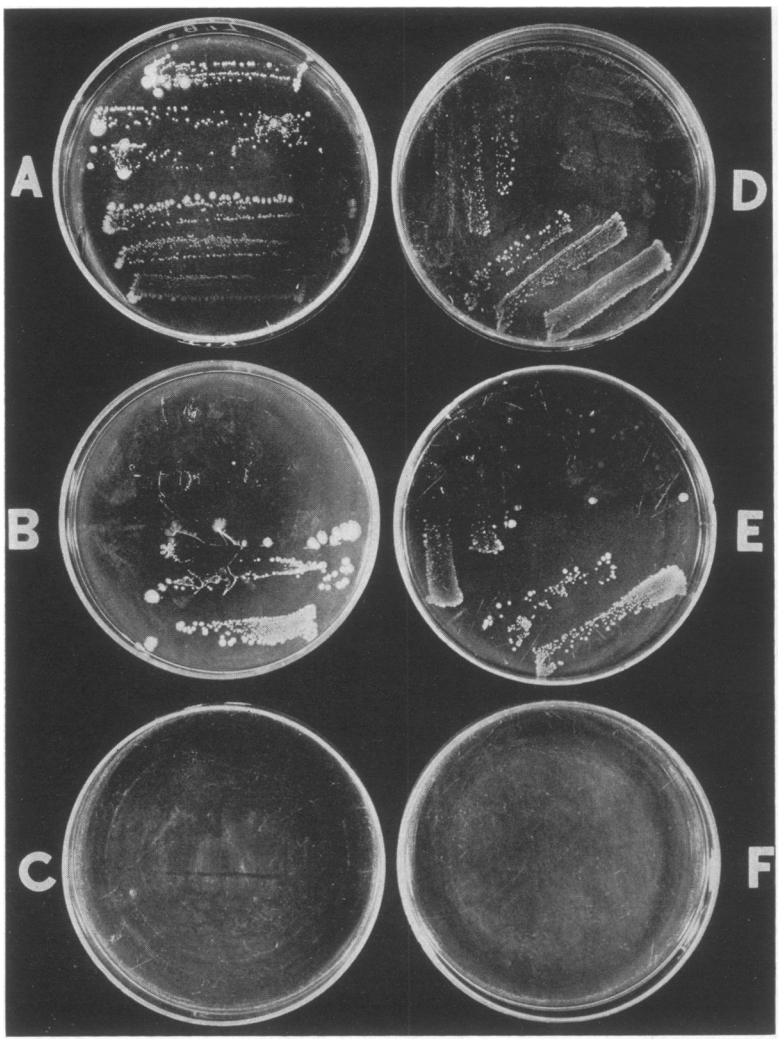

FIG. 5. INHIBITING EFFECT OF ACTINOMYCIN UPON THE GROWTH OF VARIOUS BACTERIA

A, D—Control; B, E—1 cc.; C, F—2 cc. of actinomycin suspension per plate. A, B, C: Upper—*B. abortus;* lower—*A. aerogenes.* D, E, F: Upper right—*E. coli;* upper left—intermediate; lower—*A. aerogenes.*

amounts of the crude active substance isolated from the antagonists. One ml. of the actinomycin containing about 0.4 mgm. of the crude active substance killed all cells in 24 hours; it also

killed in that period of time, all the cells in twice the above concentration (68 million viable cells); however, when five times the

TABLE 8

Bactericidal effect of various concentrations of the active substance of two antagonists upon Escherichia coli

Millions of viable bacteria*

NATURE OF ANTAGONIST	AMOUNT OF ACTIVE SUBSTANCE	TIME OF ACTION			
		3 hours	7 hours	24 hours	48 hours
	ml.†				
0	0	216	231	184	238
Actinomyces sp.....	0.01	224	264	233	154
Actinomyces sp.....	0.10	250	196	91	22
Actinomyces sp.....	1.00	126	44	0	0
P. aeruginosa.....	0.10	265	276	248	275
P. aeruginosa.....	1.00	177	205	81	93

* As determined by plating on Endo medium.

† 1 ml. = 0.4 mgm. of crude active substance.

TABLE 9

Bactericidal effect of the active substance upon growing cultures of the test organisms

GROWTH OF CULTURE BEFORE ADDITION OF ANTAGONIST	ACTION OF ANTAGONIST											
	24 hours*						48 hours*					
	Actinomyces			*P. aeruginosa*			*Actinomyces*			*P. aeruginosa*		
	E. coli	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*	*E. coli*	*B. mycoides*	*S. lutea*
hours												
Control	3†	3	3	3	3	3	3	3	3	3	3	3
0	0	0‡	0	3	0‡	0	0	0‡	0	3	0‡	0
3	0	0	0	3	0	0	0	0	0	3	0	0
6	0	0	0	3	0	0	0	0	0	3	0	0
22	3	1	0	3	1	3	0	0	0	3	0	1

* Since first inoculation, namely 0 hours.

† 0 = sterile, 1 = trace of growth, 3 = excellent growth.

‡ Some colonies formed on streak due to survival of spores.

concentration was used, not all the *Brucella* cells were killed unless double the concentration of the antagonist was used. The active substance of *Pseudomonas aeruginosa* was less effective.

A study was now made of the effect of the active substance upon actively growing cultures of the three test organisms. One-milliliter portions of the actinomycin (0.4 mgm.) and bacterial substance were added to 10 ml. nutrient broth inoculated with the different organisms. The results (table 9) show that the actinomycin killed *Sarcina lutea* and *Escherichia coli*, as well as the vegetative cells of *Bacillus mycoides* (spores remaining unaffected), when added at the start, and after 3, 6 and 22 hours inoculation with the test organisms. The bactericidal action was demonstrated by streaking the culture on nutrient agar. The substance obtained from *Pseudomonas aeruginosa* was less effec-

TABLE 10

Effect of the active substance of two antagonists upon the bacteria in fresh milk

Bacterial numbers in 1 ml. milk

NATURE OF ANTAGONIST	AMOUNT OF ACTIVE SUBSTANCE ADDED TO 10 ML. FRESH MILK	TIME OF INCUBATION	
		6 hours	16 hours
	ml.		
0	0	240,000	485,600,000
Actinomyces sp.	0.1	60,000	
Actinomyces sp.	1.0	15,000	
Actinomyces sp.	1.0		18,600,000
P. aeruginosa	0.1	160,000	
P. aeruginosa	1.0	5,000	

tive, its action being limited, killing only the *Sarcina lutea* and the vegetative cells of *Bacillus mycoides*, but not affecting *Escherichia coli*.

In order to illustrate the selective bactericidal effect of the two active substances upon a mixed bacterial population, varying amounts of these substances were added to 10-ml. portions of fresh milk. These were incubated, for 6 and 16 hours, at 28°C. and counts made, by plating on nutrient agar. The results (table 10) show that both active substances had a marked effect, even in very low concentrations, in killing and in inhibiting the multiplication of certain types of bacteria in the milk. When

0.1 and 1.0-ml. portions of actinomycin were added to 10-ml. portions of nutrient agar and fresh soil plated out, using these media, the reduction in the number of bacterial and actinomyces colonies was 97–99 per cent for the lower concentration and 100 per cent for the higher. The fungi of the soil did not seem to be affected.

SUMMARY

The soil contains a number of different types of microorganisms antagonistic to various bacteria belonging to the gram-positive and gram-negative groups. By enriching the soil with the specific bacteria, the corresponding antagonists increase and can be readily isolated. This has been done most readily by the use of an agar medium containing viable cells of the specific organism as the sole available nutrient.

Out of a number of antagonistic organisms isolated from the soil, two were studied in greater detail, one a bacterium belonging to the *Pseudomonas aeruginosa* group, and the other an *Actinomyces*. These organisms were found to inhibit the growth of several gram-negative bacteria, as well and, even to a greater extent, of numerous gram-positive bacteria.

The active substance produced by the two antagonists was found to be largely thermostable; it passed through a Seitz filter, it was removed by charcoal and was, partly at least, ether-soluble. Highly active preparations were obtained which inhibited, in very dilute solutions, the growth of *Escherichia coli*, *Brucella abortus*, and of many other bacteria.

The active substance had also a strong bactericidal effect upon *Escherichia coli* and *Brucella abortus*; 1 ml. of the preparations containing 0.4 mgm. of the crude active substance killed aqueous suspensions of *Escherichia coli* (215,000,000 viable cells) and of *Brucella abortus* (68,000,000 viable cells).

The active substance of the two antagonists was found to reduce, in very low concentrations, the bacterial population of natural substrates, such as milk; when added to agar it prevented the development of the great majority of soil bacteria and actinomycetes, but not of fungi.

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